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(54) Title: MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)

(57) Abstract

It has been found by a screening assay that XET activity is produced by an overwhelming array of phylogenetically dispersed microorganisms. Accordingly, the present invention relates to a xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism expressing an XET.

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MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)

FIELD OF THE INVENTION

The present invention relates to microbial xyloglucan endotransglycosylases (XETs) and the production and uses thereof.

BACKGROUND OF THE INVENTION

Xyloglucan endotransglycosylase (XET) is an enzyme known from plants. To our best knowledge XET has never been described from microorganisms.

Stephen C. Fry et al. suggest in Biochem. J (1992) 282, p. 821-828 that XET is responsible for cutting and rejoining intermicrofibrillar xyloglucan chains and that XET thus causes the wall-loosening required for plant cell expansion.

XET has been suggested for use in regulating the morphology of a plant, see EP 562 836 p. 2 l. 27-28.

XET is believed to be present in all plants, in particular in all land plants. XET has been extracted from dicotyledons, monocotyledons, in particular graminaceous monocotyledons and liliaceous monocotyledons, and also from a moss and a liverwort, for reference see Biochem. J (1992) 282, p. 823.

In the copending patent application PCT/DK 96/00538 (WO 97/23683) we have shown that a cellulosic material may get improved strength properties and/or improved shape-retention properties and/or improved anti-wrinkling properties after treatment with an XET enzyme.

The XET enzyme has some very interesting applications, so it is an object of the present invention to provide microbially derived xyloglucan endotransglycosylases useful for, e.g., the applications described above. The microbially derived xyloglucan endotransglycosylases would have the great advantage that they could easily be produced in great quantities.

BRIEF DISCLOSURE OF THE INVENTION

It has been found by a screening assay that XET activity is produced by an overwhelming array of phylogenetically dispersed microorganisms.

Accordingly, the present invention relates to a xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism expressing an XET; in particular the present invention relates to:

A method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising
(a) culturing in a suitable nutrient medium a microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and
(b) subsequently recovering of the XET enzyme from the nutrient medium.

Further, the present invention relates to use of the XET preparation of the present invention for treating cellulosic material, and to microbial XET preparations as such.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawing, in which

Fig. 1 shows the pH profiles of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* XET activity (Δ:*Tiarosporella phaseolina*; □:*Pseudoplectania nigrella*; and ♦:*Dichotomocladium hesseltinei*) obtained according to Example 5.

Fig. 2 shows the pH profiles of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* xyloglucanase activity (Δ:*Tiarosporella phaseolina*; □:*Pseudoplectania nigrella*; and ♦:*Dichotomocladium hesseltinei*) obtained according to Example 5.

DETAILED DISCLOSURE OF THE INVENTION

The present invention relates to a xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism.

It has been shown in the present application that XET is produced by fungi and bacteria, and it is contemplated that XET is also produced by yeasts.

An "XET-paper" was used for identifying XET producing microorganisms. The "XET-paper" is described in the copending patent application PCT/GB96/02351 (WO 97/11193); it is a xyloglucan-coated paper which is dipped through a labelled oligosaccharide.

The labelled oligosaccharide, the xyloglucan-impregnated paper, and the dot-blot test for XET activity were made in the following way:

Preparation of labelled oligosaccharide

The reducing oligosaccharide 4-O-[4-O-[4-O-[6-O-α-D-Xylopyranosyl-β-D-glucopyranosyl]-6-O-(2-O-β-D-galactopyranosyl)-α-D-xylopyranosyl-β-D-glucopyranosyl]-6-O-(2-O-β-D-galactopyranosyl)-α-D-xylopyranosyl-β-D-glucopyranosyl]-D-glucose ("XLLG") (1 gram) is dissolved in 25 ml of a saturated aqueous solution of ammonium hydrogencarbonate containing 1 gram of sodium cyanoborohydride ($NaCNBH_3$) and incubated in the dark at 25°C for 7 days to permit reductive amination. The ammonium hydrogencarbonate is then removed by drying, and the (ninhydrin-reactive) aminated derivative of XLLG is purified e.g. by gel-permeation chromatography or cation-exchange chromatography. The product is believed to be an oligosaccharidyl-1-amino-1-deoxyalditol, i.e. a derivative of XLLG in which the reducing terminal D-glucose moiety has been replaced by 1-amino-1-deoxy-D-glucitol.

The oligosaccharidyl-1-amino-1-deoxyalditol (50mg) is dissolved in 3 ml of 3% borax (di-sodium tetraborate; pH = 9.0-9.5) and a freshly-prepared solution of 10 mg lissamine rhodamine sulphonyl chloride [purchased from Molecular Probes Inc., USA] in 0.75 ml of dry dimethylformamide (DMF) is added gradually, with stirring, and the mixture is incubated in the dark overnight. A further 0.75 ml of DMF containing 10 mg lissamine rhodamine sulpohnyl chloride is added and the mixture incubated for a further 8 h. The bright pink oligosaccharidyl-1-amino-1-deoxyalditol-lissamine-rhodamine conjugate (XLLGol-SR) is purified by gel-permeation chromatography followed by reversed-phase chromatography on a C_{18}-silica gel column. After washing of the latter column with water, a methanol gradient is applied and the XLLGol-SR elutes in about 50% methanol.

Preparation of xyloglucan-impregnated paper

Whatman No.1 filter paper is moistened with a 1% aqueous solution of xyloglucan and dried. The XLLGol-SR preparation is diluted into enough 75% aqueous acetone to give an absorbance at 580 nm (A_{580}) of 0.2; the xyloglucan-coated sheet of Whatman No. 1 paper is then dipped through this solution and re-dried; the product is referred to as "XET-paper". Suitably sized pieces (e.g. 72 x 108 mm) of the XET-paper may then be glued with a non-aqueous adhesive onto a non-absorbent medium such as a sheet of transparent acetate.

Dot-blot test for XET activity

(i) A spot of the solution to be tested for XET-activity is pipetted on to a marked position in a piece of XET-paper. If the spots are 4 µl, the spacing between the samples can conveniently be 9 mm (centre-to-centre, i.e. as in a standard 96-well test plate format).

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(ii) The XET paper is then quickly (before the spots have dried) clamped between two sheets of plastic (e.g. acetate sheets, as used on overhead projectors) and incubated e.g. at 20°C for 1 hour.

(iii) The incubated XET-paper and its plastic backing is then placed (paper-side down) in a dish containing about 150 ml of a solvent [e.g. freshly prepared ethanol/formic acid/water (1:1:1 by volume)] that will remove from the paper the unreacted XLLGol-SR but not any XLLGol-SR that has become incorporated into the xyloglucan owing to XET-catalysed transglycosylation. The paper now readily detaches from the plastic backing.

(iv) The paper is then rinsed in running water for 5 minutes, then in approximately 100 ml of acetone for 5 minutes, and then dried thoroughly. If desired, drying can be expedited by a 5-minute treatment in an oven at 80°C.

(v) The paper is then examined under a short-wavelength ultraviolet lamp (e.g. emitting at 254 nm; suitable eye- and skin-protection should be worn). Active XET is indicated by a pink (orange-fluorescing) spot, which can be quantified, e.g. by use of a scanning spectrofluorimeter.

XET enzymes

We have discovered that microbial enzymes with XET activity may be either transglycosylases (meaning that they lack hydrolase activity or only have a very low hydrolase activity) or they may catalyze both transglycosylation and hydrolysis of xyloglucan.

XET enzymes with both transglycosylating and hydrolytic activities are also described for XET enzymes obtained from plants (see Annu. Rev. Plant Physiol. Plant Mol. Biol.1995.46: p. 509).

Taxonomic classification

The taxonomic classification used herein builds primarily on the system used in the :NIH Data Base (Entrez, version spring 1996) available on World Wide Web: (http://www3.ncbi.nlm.nih.gov/htbin/ef/entrezTAX).

Regarding classification of organisms which are not included in the Entrez data base the following generally available and world wide accepted reference books have been used:

For Ascomycetes: Eriksson, O.E. & Hawksworth, D.L.: Systema Ascomycetum vol 12 (1993).

For Basidiomycetes, Jülich, W.: Higher Taxa of Basidiomycetes, Bibliotheca Mycologia 85, 485pp (1981).

For Zygomycetes: O'Donnell, K.:Zygomycetes in culture, University of Georgia, US, 257pp (1979).

General mycological reference books:

Hawksworth, D.L., Kirk, P.M., Sutton, B.C. and Pegler, D.N.: Dictionary of the fungi, International Mycological Institute, 616 pp (1995);

Von Arx, J. A.: The genera of fungi sporulating in culture, 424 pp (1981).

Preferred fungi

In a preferred embodiment of the present invention the XET preparation is produced by cultivation of a fungus, in particular a fungus which belongs to *Basidiomycota*, *Zygomycota*, *Ascomycota* or a Mitosporic fungus.

A preferred *Basidiomycota* strain is a *Hymenomycetes* strain belonging to the orders *Coriolales, Schizophyllales, Stereales or Xenasmatales;* in particular a strain belonging to one of the families *Coriolaceae, Corticiaceae, Schizophyllaceae, Stereaceae* or *Tubulicrinaceae*. Preferred genera is one of the following: *Trametes, Corticium, Schizophyllum,* or *Tubulicrinis*. Preferred species is one of the following *Trametes hirsuta, Corticium roseum, Schizophyllum sp, Stereum hirsutum* or *Tubulicrinis*

subulatus.

Preferred *Ascomycota* are strains belonging to the classes *Loculoascomycetes*, *Discomycetes*, *Pyrenomycetes*, and *Plectomycetes*, preferably those belonging to the orders *Dothideales*, *Rhytismatales*, *Pezizales*, *Leotiales*, *Xylariales*, *Hypocreales*, *Halosphaeriales* , *Phyllachorales*, *Diaporthales* and *Eurotiales*.

Preferred strains are strains belonging to the families *Botryosphaeriaceae*, *Dothioraceae*, *Mycosphaerellaceae*, *Tubeufiaceae*, *Pleosporaceae*, *Leptosphaeriaceae*, *Rhytismataceae*, *Sarcosomataceae*, *Pyronemataceae*, *Ascobolaceae*, *Sclerotiniaceae*, *Amphisphaeriaceae*, *Xylariaceae*, *Hypocreaceae*, *Halosphaeriaceae*, *Phyllachoraceae*, *Valsaceae*, *Melanconidaceae* and *Trichocomataceae*; especially strains belonging to the genera *Diplodia*, *Plowrightia*, *Phyllosticta*, *Septoria*, *Tubeufia*, *Alternaria*, *Coniothyrium*, *Phoma*, *Embellisia*, *Tiarosporella*, *Galiella*, *Pseudoplectania*, *Pyronema*, *Oedocephalum*, *Botrytis*, *Aposphaeria*, *Pestalotia*, *Pestalotiopsis*, *Poronia*, *Nodulisporium*, *Xylaria*, *Fusarium*, *Verticillium*, *Volutella*, *Chaetapiospora*, *Lulworthia*, *Colletotrichum*, *Cytospora*, *Discula*, *Phomopsis*, *Coryneum*, *Seimatosporium*, *Aspergillus*, *Eurotium*, *Eupenicillium*, *Penicillium*, *Petromyces* and *Talaromyces*.

Preferred are the species *Diplodia gossypina*, *Plowrightia ribesia*, *Phyllosticta sp*, *Septoria sp*, *Tubeufia amazonensis*, *Alternaria sp*, *Embellisia hyacinthi*, *Phoma neoloba*, *Phoma tropica*, *Coniothyrium sp*, *Coniothyrium olivaceoum*, *Coniothyrium dunckii*, *Tiarosporella phaseolina* , *Tiarosporella sp*, *Galiella celebica*, *Pseudoplectania nigrella*, *Pyronema domesticum*, *Oedocephalum sp*, *Botrytis cinerea*, *Aposphaeria sp*, *Pestalotia sp*, *Pestalotiopsis sp*. *Poronia punctata*, *Xylaria sp*, *Nodulisporium sp*, *Fusarium solani*, *Verticillium sp*, *Volutella buxi*, *Chaetapiospora rhododendri*, *Lulworthia uniseptata*, *Colletotrichum aculatum*, *Colletotrichum crassipes*, *Cytospora spp*,

Discula sp, Phomopsis ilicis, Phomopsis cirsii, Coryneum castaneicola, Seimatosporium lichenicola, Aspergillus tamarii, Eurotium chevalieri, Eupenicillium javanicum, Penicillium capsulatum, Penicillium olsonii, Penicillium pinophilum, Penicillium roqueforti, Penicillium italicum, Penicillium canescens, Penicillium verruculosum, Petromyces alliaceus and *Talaromyces flavus*.

Examples of useful *Zygomycota* are strains belonging to the order *Mucorales*, preferably strains belonging to the families *Chaetocladiaceae* and *Mucoraceae*.

Preferred strains belong to the genera *Dichotomocladium, Actinomucor, Gongronella, Sporodiniella* and *Mucor*, in particular *Dichotomocladium hesseltinei, Actinomucor elegans, Gongronellla butleri, Sporodiniella umbellata* and *Mucor miehei var minor*.

Example of a strain of uncertain taxonomy is *Vialaea insculpta*.

Examples of strains belonging to the Mitosporic Fungi are *Acrodontium crateriforme, Aureobasidium pullulans, Circinotrichum sp, Cryptocline sp. Ellisiopsis sp, Epicoccum nugrum, Gliocladium sp, Helicorhoidion irregulare, Hendersonia spp, Mariannaea sp, Microsphaeropsis sp, Ramularia sp, Sarcopodium sp, Spadicoides sto, Speiropsis pedatospora, Sporotrichum exile, Stilbella sp, Trichothecium sp, Trimmatostroma abietes, Tubakia dryina, Wiesneriomyces sp* and *Zygosporium masonii*.

Preferred bacteria

In another aspect, the invention relates to a novel XET preparation which is producible by cultivation of a bacterium.

Preferred bacteria are gram-negative or gram-positive.

Examples of XET-producing gram-positive bacteria are strains belonging to the genus *Bacillus*.

Preferred strains

The following strains have been found to be XET-positive:

1. *Dichotomocladium hesseltinei*. Acc No of strain: CBS 164.61. Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

2. *Actinomucor elegans*. Ex of Acc No of strain: CBS 154.86. Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

3. *Mucor miehei var minor*. Acc No of strain: ATCC 36018. Classification: *Zygomycota, Mucorales, Mucoraceae*.

4. *Gongronella butleri*. A strain of *Gongronella butleri* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 448.97. Classification: *Zygomycota, Mucorales, Chaetocladiaceae*.

5. *Sporodiniella umbellata*. Acc No of strain: CBS 195.77. Classification: *Zygomycota, Mucorales, Mucoraceae*.

6. *Phyllosticta sp*. Isolated from a leaf of *Pithecolobium sp* from China. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Mycosphaellaceae*.

7. *Septoria sp*. A strain of *Septoria sp* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 2 January 1996, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 831.95.

Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Mycosphaellaceae.*

8. *Diplodia gossypina*. A strain of *Diplodia gossypina* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 12 March 1996, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 274.96. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Botrysphaeriaceae.*

9. *Plowrightia ribesia*. Isolated from Ribes sp., Denmark. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Dothioraceae.*

10. *Tubeufia amazonensis*. Acc No of Strain: ATCC 42524. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Tubeufiaceae.*

11. *Alternaria sp*. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Pleosporaceae.*

12. *Embellisia hyacinthi*. Acc No of species IMI 211561. Classification: *Ascomycota, Loculoascomycetes, Dothidiales, Pleosporaceae.*

13. *Phoma neoloba*. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Pleosporaceae.*

14. *Phoma tropica*. Ex on Acc No of species CBS 537.66. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Pleosporaceae.*

15. *Coniothyrium sp*. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Leptosphaeriaceae.*

16. *Coniothyrium olivaceoum*. Ex on Acc No of species CBS 304.68. Classification: *Ascomycota, Loculoascomycetes, Dothideales,*

Leptosphaeriaceae.

17. *Coniothyrium dunckii*. Classification: *Ascomycota, Loculoascomycetes, Dothideales, Leptosphaeriaceae*.

18. *Tiarosporella phaseolina*. A strain of *Tiarosporella phaseolina (Macrophomina sp)* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on the 28th January 1997, at Centraalbureau voor Schimmelcultures (CBS) under Accession No. CBS 446.97. Classification: *Ascomycota, Discomycetes, Rhytismatales, Rhytismataceae*.

19. *Tiarosporella sp*. Classification: *Ascomycota, Discomycetes, Rhytismatales, Rhytismataceae*.

20. *Galiella celebica*. Isolated from a sample collected in Japan. Classification: *Ascomycota, Discomycetes, Pezizales, Sarcosomataceae*.

21. *Pseudoplectania nigrella*. A strain of *Pseudoplectania nigrella* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 444.97. Classification: *Ascomycota, Discomycetes, Pezizales, Sarcosomataceae*.

22. *Pyronema domesticum*. Isolated from a sample from Norway. Classification: *Ascomycota, Discomycetes, Pezizales, Pyronemataceae*.

23. *Oedocephalum sp*. Classification: *Ascomycota, Discomycetes, Pezizales, Ascobolaceae*.

24. *Botrytis cinerea*. A strain of *Botrytis cinerea* has been deposited according to the Budapest Treaty on the International

Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 447.97. Classification: *Ascomycota, Discomycetes, Leotiales, Sclerotiniaceae*.

25. *Aposphaeria sp.* Classification: *Ascomycota, Discomycetes, Leotiales, Sclerotiniaceae*.

26. *Pestalotia sp.* A strain of *Pestalotia sp.* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 445.97. Classification: *Ascomycota, Pyrenomycetes, Xylariales, Amphisphaeriaceae*.

27. *Pestalotiopsis sp.* Classification: *Ascomycota, Pyrenomycetes, Xylariales, Amphisphaeriaceae*.

28. *Poronia punctata*. Isolated from a sample from Sweden. Classification: *Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae*.

29. *Xylaria sp.* Isolated from a leaf of the palm, *Sabal jamaicensis*, growing in Mona, Jamaica. Classification: *Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae Xylariaceae*.

30. *Nodulisporium sp.* Classification: *Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae*.

31. *Fusarium solani*. Isolated from a sample of grain of maize from India. Classification: *Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae*.

32. *Verticillium sp.* A strain of *Verticillium sp* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of

Patent Procedures, on 2 January 1996, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 830.95. Classification: *Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae*.

33. *Volutella buxi*. Acc No of Strain: IMI 049467. Classification: *Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae*.

34. *Chaetapiospora rhododendri*. Classification: *Ascomycota, Pyrenomycetes, Xylariales, Xylariaceae, Hyponectriaceae*.

35. *Lulworthia uniseptata*. A strain of *Lulworthia uniseptata* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 442.97. Classification: *Ascomycota, Pyrenomycetes, Halosphaeriales, Halosphaeriaceae*.

36. *Colletotrichum aculatum*. Classification: *Ascomycota, Pyrenomycetes, Phyllachorales, Phyllachoraceae*.

37. *Colletotrichum crassipes*. Classification: *Ascomycota, Pyrenomycetes, Phyllachorales, Phyllachoraceae*.

38. *Cytospora sp*. A strain of *Cytospora sp* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 23 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 424.97. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae*.

39. *Cytospora sp*. A strain of *Cytospora sp* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 23 January 1997, at Centraalbureau voor

Schimmelcultures (CBS), under Accession No. CBS 425.97. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae*.

40. *Discula sp*. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae*.

41. Phomopsis ilicis. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae*.

42. Phomopsis cirsii. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Valsaceae*.

43. *Coryneum castaneicola*. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Melanconidaceae*.

44. Seimatosporium lichenicola. Classification: *Ascomycota, Pyrenomycetes, Diaporthales, Melanconidaceae*.

45. *Aspergillus tamarii*. Ex of Acc No of strain: CBS 821.72. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

46. *Eurotium chevalieri*. Ex of Acc No of strain: CBS 472.91. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

47. *Penicillium capsulatum*. Ex of Acc No of strain: CBS 273.86. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

48. *Penicillium olsonii*. Ex of Acc No of strain: CBS 523.89. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

49. *Penicillium pinophilum*. Ex of Acc No of strain: CBS 440.89. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

50. *Penicillium roqueforti*. Ex of Acc No of strain: CBS 167.91. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

51. *Penicillium italicum*. Ex of Acc No of strain: IMI 078 681. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

52. *Penicillium canescens*. Ex of Acc No of strain: CBS 579.70. Isolated from a salt mine in Egypt. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

53. *Eupenicillium javanicum*. Ex of Acc No of the strain: CBS 448.74. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

54. *Penicillium verruculosum*. Ex of Acc No of strain: CBS 563.92. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

55. *Talaromyces flavus*. Acc No of the strain: ATCC 52201. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

56. *Petromyces alliaceus*. Acc No of strain: CBS 511.69. Classification: *Ascomycota, Plectomycetes, Eurotiales, Trichocomataceae*.

57. *Trametes hirsuta*. Isolated from a sample collected in Denmark. Classification: *Basidiomycota, Hymenomycetes, Coriolales, Coriolaceae*.

58. *Schizophyllum sp*. A strain of *Schizophyllum sp* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 28 January 1997, at Centraalbureau voor Schimmelcultures (CBS), under Accession No. CBS 443.97.

Classification: *Basidiomycota, Hymenomycetes, Schizophyllales, Schizophyllaceae.*

59. *Corticium roseum.* Isolated from a sample collected in Denmark. Classification: *Basidiomycota, Hymenomycetes, Aleurodiscales Cortiaceae.*

60. *Tubulicrinis subulatus.* Isolated from a sample collected in Denmark. Classification: *Basidiomycota, Hymenomycetes, Xenasmatales, Tubulicrinaceae.*

61. *Stereum hirsutum.* Isolated from a sample collected in Denmark. Classification: *Basidiomycota, Hymenomycetes, Stereales, Stereaceae.*

62. *Acrodontium crateriforme.* Classification: *Mitosporic fungus.*

63. *Aureobasidium pullulans.* Classification: *Mitosporic fungus.*

64. *Circinotrichum sp.* Classification: *Mitosporic fungus.*

65. *Cryptocline sp.* Classification: *Mitosporic fungus.*

66. *Ellisiopsis sp.* Classification: *Mitosporic fungus.*

67. *Epicoccum nigrum.* Classification: *Mitosporic fungus.*

68. *Gliocladium sp.* Classification: *Mitosporic fungus.*

69. *Helicorhoidion irregulare.* Classification: *Mitosporic fungus.*

70. *Hendersonia sp.* Classification: *Mitosporic fungus.*

71. *Mariannaea sp.* Classification: *Mitosporic fungus.*

72. *Microsphaeropsis sp.* Classification: *Mitosporic fungus.*

73. *Ramularia sp.* Classification: *Mitosporic fungus.*

74. *Sarcopodium sp.* Classification: *Mitosporic fungus.*

75. *Spadicoides sto.* Acc No of strain IMI203428. Classification: *Mitosporic fungus.*

76. *Speiropsis pedatospora.* Classification: *Mitosporic fungus.*

77. *Sporotrichum exile.* Acc No of strain CBS 350.47. Classification: *Mitosporic fungus.*

78. *Stilbella sp.* Classification: *Mitosporic fungus.*

79. *Trichothecium sp.* Classification: *Mitosporic fungus.*

80. *Trimmatostroma abietes.* Classification: *Mitosporic fungus.*

81. *Tubakia dryina.* Classification: *Mitosporic fungus.*

82. *Wiesneriomyces sp .* Classification: *Mitosporic fungus.*

83. *Zygosporium masonii.* Classification: *Mitosporic fungus.*

84. *Vialaea insculpta.* Classification: Uncertain.

85. *Bacillus alcalophilus.* A strain of *Bacillus alcalophilus* has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 12 February 1997, at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, under Accession No. DSM 11404.

Production of XET

The XET enzyme of the invention may be produced by aerobic cultivation of the above mentioned microbial strains on a nutrient medium containing suitable carbon and nitrogen sources, such media being known in the art.

Alternatively, the XET enzyme of the invention may be produced by aerobic cultivation of a transformed host organism containing the appropriate genetic information from, e.g., one of the above mentioned strains. Accordingly, the present invention also relates to a method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising

(a) culturing in a suitable nutrient medium a transformed host microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and

(b) subsequently recovering of the XET enzyme from the nutrient medium.

Such transformants can be prepared and cultivated by methods known in the art:

Cloning a DNA Sequence Encoding XET

The DNA sequence encoding a XET enzyme of the

invention may be isolated from any microorganism producing the XET in question, using various methods well known in the art.

First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the XET to be studied. Then, if the amino acid sequence of the XET is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify XET-encoding clones from a genomic library or cDNA prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known XET gene could be used as a probe to identify XET-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying XET-encoding clones would involve inserting fragments of genomic DNA or cDNA into an expression vector, such as a plasmid, transforming XET-negative host organism with the resulting DNA library, then plating the transformed cells onto agar plates and by use of the assay described above allowing clones expressing the XET to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers in Tetrahedron Letters 22, 1981, pp. 1859-1869 or the method described by Matthes et al. in The EMBO J. 3, 1984, pp. 801-805. In the phosphoamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. in Science 239, 1988, pp. 487-491.

Expression of XET

According to the invention, a XET-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding a XET enzyme of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a XET of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus Amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host,

examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the XET enzyme of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular.

Procedures suitable for constructing vectors of the invention encoding a XET enzyme and containing the promoter, terminator and other elements, respectively, are well known to persons skilled in the art (cf., for instance, Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA

construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a XET of the invention. The cell may be transformed with the DNA construct of the invention encoding the XET conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g., *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of

Aspergillus host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing a XET enzyme of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the XET and recovering the XET from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the XET of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

The XET secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

According to the present invention a cellulosic material may get improved strength properties and/or improved shape-retention properties and/or improved anti-wrinkling properties after treatment with a XET enzyme. The XET enzyme has the ability to rearrange and link the xyloglucan molecules which are hydrogen bonded to the cellulosic fibres whereby the above mentioned features may be achieved.

In order to enhance the effect of the XET enzyme it may in some cases be an advantage to add xyloglucan to the cellulosic material whereby the enzyme may be able to link more cellulosic material together.

The treatment of the cellulosic material with the XET enzyme may be carried out in water, or it may be carried out in water in the presence of certain components such as a buffer and/or a wetting agent and/or a stabilizer and/or a polymer

[redacted]

and/or an organic component reducing the water activity such as DMSO.

The buffer may suitably be a phosphate, borate, citrate, acetate, adipate, triethanolamine, monoethanolamine, diethanolamine, carbonate (especially alkali metal or alkaline earth metal, in particular sodium or potassium carbonate, or ammonium and HCl salts), diamine, especially diaminoethane, imidazole, Tris or amino acid buffer.

The wetting agent serves to improve the wettability of the cellulosic material. The wetting agent is preferably of a non-ionic surfactant type.

The stabilizer may be an agent stabilizing the XET enzyme.

According to the invention the concentration of XET in the aqueous medium may be 0.01-1000 µg of enzyme protein per g cellulosic material, preferably 0.05-100 µg of enzyme protein per g cellulosic material.

It will generally be appropriate to incubate the reaction medium (containing the cellulosic material and the XET enzyme) for a period of at least a few minutes. An incubation time of from 1 minute to 20 hours will generally be suitable, in particular an incubation time of from 30 minutes to 10 hours will often be preferred.

The temperature of the reaction medium in the process of the invention may suitably be in the range of 10-90°C, in particular in the range of 15-70°C, as appropriate for the XET enzyme in question.

The invention is further illustrated in the following non-limiting examples.

EXAMPLE 1

Screening for positive XET strains.

Media

PD Agar: 39 g potato dextrose agar, DIFCO 0013; add deionized water up to 1000 ml,
autoclave at 121°C for 15 - 20 min.

YPG agar: 4 g yeast extract (DIFCO 0127),

1 g KH_2PO_4 (Merck 4873),

0.5 g $MgSO_4 . 7H_2O$ (Merck 5886)

15 g dextrose (Roquette 101-0441)

20 g agar (Merck 1614)

deionized water up to 1000 ml

Autoclave at 121° C for 15 - 20 min.

MEA: 20 g malt extract powder (Difco 0186)

1 g peptone (Difco 0118)

20 g glucose (Roquette France 1010441)

20 g agar (Merck 1614)

deionized water up to 1000 ml

Autoclave at 121°C for 15 min

Medium A. Per flask: 30 g wheat bran , 45 ml of the following solution: 10 g rofec (Roquette 101-0441), 10 g NH_4NO_3 (Merck 1187), 10 g KH_2PO_4 (Merck 4873), 40 g Solcafloc (Dicacel availabe from Dicalite-Europe-Nord, 9000 Gent, Belgium), 0.75g $MgSO_4.7H_2O$ (Merck 5886), 15 g $CaCO_3$, tap water to 1000 ml, pH adjusted to 6.5.

Autoclave for 40 min at 121° C.

Medium B. 20 g soyabean meal, 5 g maltodex 01, (Roquette 101-7845), 15 g wheat bran, 3 g peptone (Difco 0118), 10 g cellulose avicel (Merck 2331), 0.2 ml pluronic (PE-6100, 101-3068), 1 g olive oil, deionized water up to 1000 ml.

100 ml in 500 ml Erlenmeyer flask with 2 baffles. Autoclave at 121° C for 40 min.

Medium C. 100 g sucrose, 40 g soybean meal, 10 g $Na_2HPO_4.12H_2O$ (Merck 6579), 0.1 ml pluronic (PE 6100), tap water up to 1000 ml. 0.5 g $CaCO_3$ in a 2 baffled Erlenmeyer flask with 100 ml medium. Autoclave at 121°C for 40 minutes. Just prior to use, add 10 ml of 1M $NaHCO_3$ pH 9 per 100 ml medium.

FERMENTATION PROCEDURE

The fungal strains were maintained on agar in petri dishes (9 cm) or on slants with PDA, YPG or MEA (see list of media). 1 agar slant was washed off with 10 ml sterile distilled water and 3 ml were used to inoculate 1 shake flask.

The fungal strains were grown in shake flasks under the following growth conditions:

media: A and B (see list of media)

Temperature: 26°C

RPM: A : stationary

B : 125 - 200

Incubation time : A : 6 to 30 days

B: 2 - 21 days.

The bacterial isolate was maintained on agar slopes with TY agar pH 9 which consists of:

20 g tryptone, 5 g yeast extract, 0.7 ml of a 1% solution of $FeCl_2 . 4H_2O$, 0.1 ml of a 1% solution of $MnCl_2 . 4H_2O$, 1.5 ml of a 1% solution of $MgSO_4 . 7H_2O$, 20 g agar and deionized water up to 1000ml. Autoclave at 121°C for 20 minutes. Before pouring the plates, add 10 ml of sterile 1M $NaHCO_3$ pH9 to 100 ml agar.

The slants were incubated at 30°C for 4 days. 1 slant was washed off with 10 ml sterile distilled water and 3 ml were used to inoculate each flask.

The bacterium was grown in shake flasks containing Medium C at 30°C for 4 days at 300 rpm.

ACTIVITY TEST:

The culture broths which were produced in B and C, were centrifuged at 10,000 rpm for 10 minutes. The supernatants were tested for XET activity.

To each flask with wheat bran and fully grown culture, was added 200 ml tap water and shaken at 200 rpm for 2 hours. The culture broth was then centrifuged and the supernatant tested for XET.

XET ANALYSIS

The samples were analysed using the "dot-blot" assay described above.

RESULTS

Using the method described above, 1 Bacillus sp and several fungi belonging to different taxonomical groups were found to be positive. The results are shown in the table below.

The *Bacillus alcalophilus* was positive when grown on Medium C pH

9 (see list of media) for 4 days at 30°C at 300 rpm.

Fungal Isolate	Medium A	Medium B
Actinomucor elegans	+	-
Dichotomocladium hesseltinei	+	+
Gongronella butleri	not done	+
Mucor miehei var minor	+	+
Sporodiniella umbellata	-	+
Alternaria sp	+	-
Aposphaeria	not done	+
Aspergillus tamarii	+	+
Botrytis cinerea	+	+
Chaetapiospora rhododendri	-	+
Colletotrichum aculatum	+	-
Colletotrichum crassipes	+	-
Coniothyrium dunkii	+	+
Coniothyrium olivaceoum	+	-
Coniothyrium sp	-	+
Coryneum castaneicola	-	+
Cytospora spp	+	+
Diplodia gossypina	+	+
Discula sp	+	+
Embellisia hyacinthi	+	-
Eupenicillium javanicum	+	-

Eurotium chevalieri	-	+
Fusarium solani	+	-
Galiella celebica	+	+
Lulworthia uniseptata	+	+
Nodulisporium sp	+	-
Oedocephalum sp	-	+
Penicillium canescens	+	-
P. capsulatum	+	-
P. italicum	not done	+
P. olsonii	+	-
P. pinophilum	+	not done
P. roqueforti	+	+
P. verruculosum	+	+
Pestalotia sp	not done	+
Pestalotiopsis sp	+	+
Petromyces alliaceus	+	+
Phoma neoloba	+	-
Phoma tropica	+	+
Phomopsis cirsii	-	+
Phomopsis ilicis	+	+
Phyllosticta sp	+	-
Plowrightia ribesia	+	+
Poronia punctata	+	+
Pseudoplectania nigrella	+	+

Pyronema domesticum	+	+
Ramularia sp	+	-
Seimatosporium lichenicola	+	-
Septoria sp	+	not done
Talaromyces flavus	+	+
Tubeufia amazonensis	+	-
Tiarosporella phaseolina	+	+
Tiarosporella sp	-	+
Verticillium sp	+	+
Volutella buxi	+	-
Xylaria sp	+	+
Corticium roseum	+	+
Schizophyllum sp	+	+
Stereum hirsutum	+	+
Trametes hirsuta	+	+
Tubulicrinis subulatus	+	+
Acrodontium crateriforme	+	-
Aureobasidium pullulans	+	-
Circinotrichum sp	-	+
Cryptocline sp	+	-
Ellisiopsis sp	+	-
Epicoccum nigrum	-	+
Gliocladium sp	+	-
Helicorhoidion irregulare	+	+

Hendersonia spp	+	+
Mariannaea sp	+	-
Microsphaeropsis sp	+	-
Ramularia sp	+	-
Sarcopodium sp	+	-
Spadicoides sp	-	+
Speiropsis pedatospora	+	-
Sporotrichum exile	+	+
Stilbella sp	+	-
Trichothecium sp	+	+
Trimmatostroma abietes	+	+
Tubakia dryina	+	+
Wiesneriomyces sp	+	-
Zygosporium masonii	+	+
Vialaea	+	not done

+ = positive, - = negative.

EXAMPLE 2

Purification and characterization of *Dichotomocladium hesseltinei* XET.

15 PDA agar slopes were inoculated with *Dichotomocladium hesseltinei* (CBS 164.61) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80 and used to inoculate 80 shake flasks containing Medium B (2 - 3ml / flask). The flasks were shaken at 200 rpm,

26°C for 5 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

The supernatant containing xyloglucan endotransglucosylase (XET) was purified using the following method:

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The pH of the filtrate was adjusted to pH 8.0 and the filtrate was diluted with deionised water to give the same conductivity as 20mM Tris/HCl, pH 8.0.

The XET enzyme was applied to a Q-sepharose FF column equilibrated in 20mM Tris/HCl, pH 8.0 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M). The XET activity eluted as a single peak. The pooled XET was transferred to 20mM Tris/HCl, pH 8.0 on a Sephadex G25 column and rechromatographed on a Q-sepharose FF column equilibrated in 20mM Tris/HCl, pH 8.0. The column was eluted with an increasing linear NaCl gradient (0 → 0.2M). XET containing fractions were pooled and $(NH_4)_2SO_4$ was added to 1.4M final concentration. A Phenyl Toyopearl 650S column was equilibrated in 1.4M $(NH_4)_2SO_4$, 10mM succinic acid, pH 7.0 and the XET enzyme was applied to this column and eluted with a decreasing linear $(NH_4)_2SO_4$ gradient (1.4 → 0M). XET containing fractions were pooled and concentrated on an ultrafiltration cell with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 100mM H_3BO_3, 10mM dimethyl glutaric acid, 2mM $CaCl_2$, pH 7.0. Fractions eluted from the Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

The *Dichotomocladium hesseltinei* XET migrates on SDS-PAGE as a band with M_r = 24 kDa. N-terminal amino acid sequencing of the 24 kDa component was carried out following SDS-PAGE and

electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence (SEQ ID No. 1) could be deduced:

Ala-Glu-Phe-Cys-Gly-Gln-Trp-Asp-Thr-Gln-Thr-Val-Gly-Asn-Tyr-Ile-Val-Tyr-Asn-Asn-Leu-Leu-Gly-Ala-Gly-Ser-Ala.

The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 1 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No.1, preferably being at least 85% homologous with SEQ ID No. 1, more preferably being at least 90% homologous with SEQ ID No.1, even more preferably being at least 95% homologous with SEQ ID No. 1, in particular being at least 98% homologous with SEQ ID No. 1.

A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

In addition, mass spec. analysis of the *Dichotomocladium hesseltinei* XET gave a value of 23 006 Da.

EXAMPLE 3

Purification and characterization of *Tiarosporella phaseolina* XET.

15 PDA agar slopes were inoculated with *Tiarosporella phaseolina* (CBS 446.97) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80 and used to inoculate 80 shake flasks containing Medium B (2 - 3ml/flask). The flasks were shaken at 200 rpm, 26°C for 7 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

The supernatant containing xyloglucan endotransglucosylase (XET)

was purified using the following method:

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The filtrate was concentrated by ultrafiltration on 3 kDa cut-off polyethersulfone membranes followed by dialfiltration with distilled water to reduce the conductivity. The pH of the concentrated enzyme was adjusted to pH 5.0. The conductivity of the concentrated enzyme was 1.7 mS/cm.

The XET enzyme was applied to a S-sepharose FF column equilibrated in 20mM CH_3COOH/NaOH, pH 5.0 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.5M). The XET activity eluted as a single peak. The pooled XET was transferred to 20mM CH_3COOH/NaOH, pH 4.0 by dialysis. The dialysed enzyme was applied to a SOURCE 30S column equilibrated in 20mM CH_3COOH/NaOH, pH 4.0. After washing the column the XET activity was eluted with an increasing linear NaCl gradient (0 → 0.5M). Fractions with XET activity were pooled and dialysed against 20mM Tris/HCl, pH 8.0. The dialysed enzyme was applied to a SOURCE 30Q column equilibrated in 20mM Tris/HCl, pH 8.0. After washing the column the XET activity was eluted with an increasing linear NaCl gradient (0 → 0.5M). Fractions with XET activity were pooled and concentrated on an ultrafiltration cell with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 100mM H_3BO_3, 10mM dimethyl glutaric acid, 2mM $CaCl_2$, pH 7.0. Fractions eluted from the Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

The *Tiarosporella phaseolina* XET migrates on SDS-PAGE as a band with M_r = 24 kDa. N-terminal amino acid sequencing of the 24 kDa component was carried out following SDS-PAGE and electroblotting onto a PVDF-membrane. The following N-terminal amino acid sequence (SEQ. ID No. 2) could be deduced:

Xaa-Asp-Phe-Cys-Gly-Gln-Trp-Asp-Asn-Val-Lys-Asn-Gly-Pro-Tyr-Thr-Leu-Tyr-Asn-Asn-Leu-Gly-Gly-Lys

The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 2 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No. 2, preferably being at least 85% homologous with SEQ ID No. 2, more preferably being at least 90% homologous with SEQ ID No. 2, even more preferably being at least 95% homologous with SEQ ID No. 2, in particular being at least 98% homologous with SEQ ID No. 2.

A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

EXAMPLE 4

Purification and characterization of *Pseudoplectania nigrella* XET.

15 PDA agar slopes were inoculated with *Pseudoplectania nigrella* (CBS 444.97) and incubated at 26°C for 7 days. They were washed off with about 250 ml sterile distilled water with 0.1% Tween 80 and used to inoculated 80 shake flasks containing Medium B (2 - 3ml/flask). The flasks were shaken at 200 rpm, 26°C for 7 days after which time the culture broth was centrifuged at 4000 rpm for 15 minutes.

The supernatant containing xyloglucan endotransglycosylase (XET) was purified using the following method:

Filter aid was added to the culture broth which was filtered through a filtration cloth. This solution was further filtered through a Seitz depth filter plate resulting in a clear solution. The pH of the filtrate was adjusted to pH 5.0 and the

In Table 1 the sequences obtained from 8 peptides are shown. Two peptides were found to be homologous to glucanase- and xylanase-like enzymes but the majority did not show any or only irrelevant homology to known sequences.

Table 1. Sequence of Lys-C peptides from *P.nigrella* XET

Peptide	Run no.	Sequence	Comments on homology
XET lysyl 070198-2fr5	3777	WNDPVVK	Homology to B. subtilis lysis protein
XET lysyl 080198-4fr3	3778	(S/Y)RFNAPALIGE[WQ]	Nothing found
XET lysyl 090198-1fr6	3779	LIFE	Too small
XET lysyl 070198-1fr5	P379	EDGSYLKYAK	irrelevant homologies
XET lysyl 070198-1fr3	P380	EWGTTGKFNK	Homology to endoglucanase
XET lysyl 080198-1fr1	P381	KVTAVEAWK	Weak homology to xyn1_asptu. Endoxylanase
XET lysyl 080198-2fr1	P382	XFYQIANS(Q/I)	Nothing found
XET lysyl 080198-3fr1	P383	AALXXVMK	Nothing found

EXAMPLE 5

pH profile of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania nigrella* XET and xyloglucanase

The xyloglucan endotransglycosylase of *Dichotomocladium hesseltinei*, *Tiarosporella phaseolina* and *Pseudoplectania*

filtrate was diluted with deionised water to give the same conductivity as 20mM CH_3COOH/NaOH, pH 5.0.

The XET enzyme was applied to a S-sepharose FF column equilibrated in 20mM CH_3COOH/NaOH, pH 5.0 and the enzyme was eluted with an increasing linear NaCl gradient (0 → 0.25M). The XET activity eluted as a single peak. The pooled XET was transferred to 20mM Hepes/NaOH, pH 7.0 on a Sephadex G25 column and applied to a Q-sepharose FF column equilibrated in 20mM Hepes/NaOH, pH 7.0. The column was eluted with an increasing linear NaCl gradient (0 → 0.5M). XET containing fractions were pooled and transferred to 20mM CH_3COOH/NaOH, pH 5.0 by dialysis. A SOURCE 15S column was equilibrated in 20mM CH_3COOH/NaOH, pH 5.0, and the dialysed enzyme was applied. After washing the column the XET enzyme was eluted with an increasing linear NaCl gradient (0 → 0.2M). XET containing fractions were pooled and concentrated on an ultrafiltration cell with a 10kDa cut-off regenerated cellulose membrane. The concentrated enzyme was applied to a Superdex200 size exclusion column equilibrated in 20mM CH_3COOH/NaOH, 100mM NaCl, pH 5.0. Fractions eluted from the Superdex200 column were analyzed by SDS-PAGE and pure XET fractions were pooled.

The *Pseudoplectania nigrella* XET migrates on SDS-PAGE as a band with M_r = 58 kDa. Following SDS-PAGE and electroblotting onto a PVDF-membrane it was found that the 58 kDa component had a blocked N-terminus.

A highly purified preparation of *Pseudoplectania nigrella* XET was reduced and alkylated. A sample of the enzyme was then degraded with Lys-C. Peptides were isolated by RP-HPLC on a long Vydac C18 in a SMART system using TFA/AN and repurified in TFA/isopropanol. Selected peptides were analyzed by Edman degradation.

EXAMPLE 6

Cloning and expression of a xyloglucan endotransglycosylase enzyme (XET) from *Tiarosporella phaseolina* CBS No. 446.97

Deposited organisms:

Tiarosporella phaseolina CBS No. 446.97.

Other strains:

Yeast strain: The *Saccharomyces cerevisiae* strain used was W3124 (van den Hazel, H.B; Kielland-Brandt, M.C.; Winther, J.R. in Eur. J. Biochem., 207, 277-283, 1992; (MATa; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-1137; prc1::HIS3; prb1:: LEU2; cir+).

E. coli strain: DH10B (Life Technologies)

Plasmids:

The *Aspergillus* expression vector pHD414 is a derivative of the plasmid p775 (described in EP 238 023). The construction of pHD414 is further described in WO 93/11249.

pYES 2.0 (Invitrogen)

Media:

YPD:

10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% glucose (sterile filtered) added.

YPM:

10 g yeast extract, 20 g peptone, H_2O to 900 ml. Autoclaved, 100 ml 20% maltodextrin (sterile filtered) added.

10 x Basal salt:

75 g yeast nitrogen base, 113 g succinic acid, 68 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA:

100 ml 10 x Basal salt, 28 ml 20% casamino acids without vitamins, 10 ml 1% tryptophan, H_2O ad 900 ml, autoclaved, 3.6 ml 5% threonine and 100 ml 20% glucose or 20% galactose added.

SC-agar:

SC-URA, 20 g/l agar added.

nigrella were checked for their pH profile. The pure enzymes were diluted in buffers ranging from pH 3.0 to 11.0 so that in the final dilution, the protein concentration was the same i.e. A_{280} = 0.004. The samples were then assayed for XET using the XET-dot-blot-assay (described earlier). The fluorescent spots were judged visually and graded from 0 - 10. The units used in Fig. 1 are therefore arbitrary units.
It can be seen from Fig. 1 that the XET is active between pH 3 and pH 11, in particular between pH 4 and pH 9.

All the isolates with XET activity were also tested for xyloglucanase activity. The ratio between the 2 enzymes varies from isolate to isolate. The enzymes were diluted in the same buffers as for the XET pH profile activity until the final protein concentration was the same and assayed for xyloglucanase activity. AZCL xyloglucan was used. The xyloglucanase method used was the following:

Substrate: 0.4% AZCL-xyloglucan suspended in demineralised water.
Buffer: 100mM H_3BO_3, 10mM Dimethy glutaric acid, 2mM $CaCl_2$, pH 7.
Analysis:
- An Eppendorf thermomixer is switched on at 40° C
- 500µl 0.4% AZCL-xyloglucan is mixed with 500µl buffer and put on ice.
- 20µl enzyme is added and incubated in the Eppendorf thermomixer until a suitable colour is reached.
- The samples are returned to the ice bath to prevent further reaction while the samples are centrifuged at 0°C.
- 200 µl samples are transferred to micro titre plates and the blue colour is measured at 650nm.

The results are shown in Fig. 2.

AZCL Xyloglucan (Megazyme, Australia)

Expression cloning in yeast

Expression cloning in yeast was done as described by H. Dalboege et al. (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953), which are hereby incorporated as reference. All individual steps of Extraction of total RNA, cDNA synthesis, Mung bean nuclease treatment, Blunt-ending with T4 DNA polymerase, and Construction of libraries was done according to the references mentioned above.

Fermentation procedure of *Tiarosporella phaseolina* CBS No. 446.97 for mRNA isolation:

Tiarosporella phaseolina CBS No. 446.97 was inoculated from a plate with outgrown mycelium into a shake flask containing 100 ml medium B (see media). The culture was incubated at 26°C and 200 rpm for 7 days. The resulting culture broth was filtered through miracloth and the mycelium was frozen down in liquid nitrogen.

mRNA was isolated from mycelium from this culture as described in (H. Dalboege et al Mol. Gen. Genet (1994) 243:253-260.; WO 93/11249; WO 94/14953).

Extraction of total RNA was performed with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion, and isolation of poly(A)$^+$RNA was carried out by oligo(dT)-cellulose affinity chromatography using the procedures described in WO 94/14953.

cDNA synthesis:

Double-stranded cDNA was synthesized from 5 mg poly(A)$^+$ RNA by the RNase H method (Gubler and Hoffman (1983) Gene 25:263-269, Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). The poly(A)$^+$ RNA (5 μg in 5 μl of DEPC-treated water) was heated at 70°C for 8 min. in a pre-siliconized, RNase-free Eppendorph tube, quenched on ice and combined in a final volume of 50 μl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, Bethesda Research Laboratories) containing 1 mM of dATP, dGTP and dTTP and 0.5 mM 5-methyl-dCTP

(Pharmacia), 40 units human placental ribonuclease inhibitor (RNasin, Promega), 1.45 μg of oligo(dT)$_{18}$-Not I primer (Pharmacia) and 1000 units SuperScript II RNase H reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour. After synthesis, the mRNA:cDNA hybrid mixture was gelfiltrated through a MicroSpin S-400 HR (Pharmacia) spin column according to the manufacturer's instructions.

After the gelfiltration, the hybrids were diluted in 250 μl second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.16 mM bNAD+) containing 200 μl of each dNTP, 60 units *E. coli* DNA polymerase I (Pharmacia), 5.25 units RNase H (Promega) and 15 units *E. coli* DNA ligase (Boehringer Mannheim). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 2 hours and additional 15 min. at 25°C. The reaction was stopped by addition of EDTA to a final concentration of 20 mM followed by phenol and chloroform extractions.

Mung bean nuclease treatment:

The double-stranded cDNA was precipitated at -20°C for 12 hours by addition of 2 vols 96% EtOH, 0.2 vol 10 M NH_4Ac, recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 μl Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM DTT, 2% glycerol) containing 25 units Mung bean nuclease (Pharmacia). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 min., followed by addition of 70 μl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction and precipitation with 2 vols of 96% EtOH and 0.1 vol 3 M NaAc, pH 5.2 on ice for 30 min.

Blunt-ending with T4 DNA polymerase:

The double-stranded cDNAs were recovered by centrifugation and blunt-ended in 30 ml T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM of each dNTP and 5 units T4 DNA polymerase (New England Biolabs) by incubating the reaction mixture at 16°C for 1 hour. The reaction was stopped by addition of EDTA to a final

concentration of 20 mM, followed by phenol and chloroform extractions, and precipitation for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Adaptor ligation, Not I digestion and size selection:

After the fill-in reaction the cDNAs were recovered by centrifugation, washed in 70% EtOH and dried. The cDNA pellet was resuspended in 25 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 2.5 µg non-palindromic BstXI adaptors (Invitrogen) and 30 units T4 ligase (Promega) and incubated at 16°C for 12 hours. The reaction was stopped by heating at 65°C for 20 min. and then cooling on ice for 5 min. The adapted cDNA was digested with Not I restriction enzyme by addition of 20 µl water, 5 µl 10x Not I restriction enzyme buffer (New England Biolabs) and 50 units Not I (New England Biolabs), followed by incubation for 2.5 hours at 37°C. The reaction was stopped by heating at 65°C for 10 min. The cDNAs were size-fractionated by gel electrophoresis on a 0.8% SeaPlaque GTG low melting temperature agarose gel (FMC) in 1x TBE to separate unligated adaptors and small cDNAs. The cDNAs were size-selected with a cut-off at 0.7 kb and rescued from the gel by use of b-Agarase (New England Biolabs) according to the manufacturer's instructions and precipitated for 12 hours at -20°C by adding 2 vols 96% EtOH and 0.1 vol 3 M NaAc pH 5.2.

Construction of library:

The directional, size-selected cDNAs were recovered by centrifugation, washed in 70% EtOH, dried and resuspended in 30 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The cDNAs were desalted by gelfiltration through a MicroSpin S-300 HR (Pharmacia) spin column according to the manufacturer's instructions. Three test ligations were carried out in 10 µl ligation buffer (30 mM Tris-Cl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP) containing 5 µl double-stranded cDNA (reaction tubes #1 and #2), 15 units T4 ligase (Promega) and 30 ng (tube #1), 40 ng (tube #2) and 40 ng (tube #3, the vector background control) of BstXI-NotI cleaved pYES 2.0 vector. The ligation reactions were performed by incubation at 16°C for 12 hours, heating at 70°C for 20 min. and

addition of 10 µl water to each tube. 1 µl of each ligation mixture was electroporated into 40 µl electrocompetent *E. coli* DH10B cells (Bethesda research Laboratories) as described (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY). Using the optimal conditions a library was established in *E. coli* consisting of pools. Each pool was made by spreading transformed *E. coli* on LB+ampicillin agar plates giving 15.000-30.000 colonies/plate after incubation at 37°C for 24 hours. 20 ml LB+ampicillin was added to the plate and the cells were suspended herein. The cell suspension was shaked in a 50 ml tube for 1 hour at 37°C. Plasmid DNA was isolated from the cells according to the manufacturer's instructions using QIAGEN plasmid kit and stored at -20°C.

1 µl aliquots of purified plasmid DNA (100 ng/ml) from individual pools were transformed into *S. cerevisiae* W3124 by electroporation (Becker and Guarante (1991) Methods Enzymol. 194:182-187) and the transformants were plated on SC agar containing 2% glucose and incubated at 30°C.

Identification of positive colonies:

Colonies were screened indirectly for XET by finding xyloglucanase positive colonies.

After 3-5 days of growth, the agar plates were replica plated onto a set of SC-URA agar (with 20% galactose added) plates containing 0.1% AZCL Xyloglucan. These plates were incubated for 3-7 days at 30°C. Xyloglanase positive colonies were identified as colonies surrounded by a blue halo.

Cells from enzyme-positive colonies were spread for single colony isolation on agar, and an enzyme-producing single colony was selected for each of the Xyloglucanase-producing colonies identified.

All xyloglucanase positive colonies were tested for XET and were found to be positive.

Isolation of a cDNA gene for expression in *Aspergillus*:

An XET-producing yeast colony was inoculated into 20 ml YPD broth in a 50 ml glass test tube. The tube was shaken for 2 days

at 30°C. The cells were harvested by centrifugation for 10 min. at 3000 rpm.

DNA was isolated according to WO 94/14953 and dissolved in 50 ml water. The DNA was transformed into *E. coli* by standard procedures. Plasmid DNA was isolated from *E. coli* using standard procedures, and analyzed by restriction enzyme analysis. The cDNA insert was excised using the restriction enzymes BamH I and Xba I and ligated into the Aspergillus expression vector pHD414 resulting in the plasmid pA2XG80.

The cDNA inset of Qiagen purified plasmid DNA of pA2XG80 (Qiagen, USA) was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and synthetic oligonucleotide primers using an Applied Biosystems ABI PRISM™ 377 DNA Sequencer according to the manufacturers instructions.

Transformation of *Aspergillus oryzae* or *Aspergillus niger*

Protoplasts are prepared as described in WO 95/02043, p. 16, line 21 - page 17, line 12, which is hereby incorporated by reference.

100 µl of protoplast suspension is mixed with 5-25 µg of the appropriate DNA in 10 µl of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$). Protoplasts are mixed with p3SR2 (an *A. nidulans* amdS gene carrying plasmid) (Tove Christensen et al. Bio/Technology, pp 1419-1422 vol.6, Dec. 1988). The mixture is left at room temperature for 25 minutes. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 minutes, spun at 2500 g for 15 minutes and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4-7 days at 37°C spores are picked and spread for single colonies. This procedure is repeated and spores of a single colony after the

second reisolation is stored as a defined transformant.

Test of *A. oryzae* transformants

Each of the *A. oryzae* transformants is inoculated in 10 ml of YPM (cf. below) and propagated. After 2-5 days of incubation at 30°C, the supernatant is removed.

The XET activity is identified by using the XET dot-blot assay described earlier.

The following sequence (SEQ ID No. 3) is the cDNA insert in pYES2.0 of XG80 (XET from Tiarosporella phaseolina) including the BamH I and Not I recognition sites used for cloning:

GGATCCGAATTCCAACTATCCTGCCCTCCTTTCAAGCGAACACCATGAAGTTCTCCTCGGCTCT
GTTTCTGGCCGCTACG
GCGGTCTTGGCTTCCGCCGCGCCGCTTGAGCGCCGCGCCGACTTTTGTGGTCAATGGGACAACG
TGAAGAACGGACCTTA
CACTCTTTACAACAACCTGTGGGGAAAAGATGCTTCCGGAGCCTCCGGATCGCAATGCACCGGC
GTCGATAGCTTCAGCA
GCAACACCATCGCTTGGCACACATCCTGGTCCTGGTCCGGTGCTCAGTACAATGTCAAGTCTTA
CGCAAACGTGGTCGTC
GACATCACCTCTAAGAAACTCAGCGCCATCAGCAGCATTAACAGCATCTGGCGCTGGGCTTACA
CGGGTAGCAACATTGT
TGCCAATGTTGCCTACGATATCTTCACTTCATCCACTGTCGGTGGTAGCGAGGAATATGAAATC
ATGATATGGGTTGGTG
CTCTCGGTGGTGCTGGTCCGATCTCATCTACCGGCTCCCCTATTGCCACCGTTTCCCTTGCAGG
CTCCTCGTGGAAGCTC
TACAAAGGGCCCAACGGGCAGATGACCGTGTTCAGCTTCGTCGCCGAGTCCAACGTGAACAACT
TCAGCGGTGACCTTAA
CGCTTTCATCAAGTACCTCACCGGCAACCAGGGCCTTCCCGCCTCGCAATACATCAAGAGCATT
GGCGCTGGCACTGAGC
CGTTCACGGGTTCCAACGCCAAGCTGACCACTTCCTCCTACACTGTCAGCTTCAGATAACTGTG
AAGCTTTATGCTGCCC
TTATGCATCATCCTTGTACATAGTTATCACCAGGGGACTCTTGTAAATACGATTGCCTTATTAA
CCGCCTGCATCTGCTT
TCACACAATGGCATTTACCAATCAACAGTGCGCCTCGAATCCGTAAAAGGTGGCTTAAAAAAAA
AAAAAAAAAAAAAAAA
AATTCCTGCGGCCGC.

The following sequence (SEQ ID no. 4) is the amino acid sequence of the coding region of XG80:

MKFSSALFLAATAVLASAAPLERRADFCGQWDNVKNGPYTLYNNLWGKDASGASGSQCTGVDSF
SSNTIAWHTSWSWSGA
QYNVKSYANVVVDITSKKLSAISSINSIWRWAYTGSNIVANVAYDIFTSSTVGGSEEYEIMIWV
GALGGAGPISSTGSPI
ATVSLAGSSWKLYKGPNGQMTVFSFVAESNVNNFSGDLNAFIKYLTGNQGLPASQYIKSIGAGT
EPFTGSNAKLTTSSYT
VSFR.

The present invention also relates to a microbial XET enzyme comprising the amino acid sequence shown in SEQ ID No. 4 or a XET being at least 80% homologous with the amino acid sequence SEQ ID No. 4, preferably being at least 85% homologous with SEQ ID No. 4, more preferably being at least 90% homologous with SEQ ID No. 4, even more preferably being at least 95% homologous with SEQ ID No. 4, in particular being at least 98% homologous with SEQ ID No. 4.

A polypeptide is considered to be X% homologous to the parent XET if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

A clone C1.XG80 was deposited at DSMZ on 24 February 1998 under Accession No. DSM 12032.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44 44 88 88
(H) TELEFAX: +45 44 49 32 56

(ii) TITLE OF INVENTION: MICROBIAL XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XET)

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Ala Glu Phe Cys Gly Gln Trp Asp Thr Gln Thr Val Gly Asn Tyr Ile
1               5                  10                  15

Val Tyr Asn Asn Leu Leu Gly Ala Gly Ser Ala
            20                  25
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Xaa Asp Phe Cys Gly Gln Trp Asp Asn Val Lys Asn Gly Pro Tyr Thr
1               5                  10                  15

Leu Tyr Asn Asn Leu Gly Gly Lys
```

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 975 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGATCCGAAT TCCAACTATC CTGCCCTCCT TTCAAGCGAA CACCATGAAG TTCTCCTCGG 60

CTCTGTTTCT GGCCGCTACG GCGGTCTTGG CTTCCGCCGC GCCGCTTGAG CGCCGCGCCG 120

ACTTTTGTGG TCAATGGGAC AACGTGAAGA ACGGACCTTA CACTCTTTAC AACAACCTGT 180

GGGGAAAAGA TGCTTCCGGA GCCTCCGGAT CGCAATGCAC CGGCGTCGAT AGCTTCAGCA 240

GCAACACCAT CGCTTGGCAC ACATCCTGGT CCTGGTCCGG TGCTCAGTAC AATGTCAAGT 300

CTTACGCAAA CGTGGTCGTC GACATCACCT CTAAGAAACT CAGCGCCATC AGCAGCATTA 360

ACAGCATCTG GCGCTGGGCT TACACGGGTA GCAACATTGT TGCCAATGTT GCCTACGATA 420

TCTTCACTTC ATCCACTGTC GGTGGTAGCG AGGAATATGA AATCATGATA TGGGTTGGTG 480

CTCTCGGTGG TGCTGGTCCG ATCTCATCTA CCGGCTCCCC TATTGCCACC GTTTCCCTTG 540

CAGGCTCCTC GTGGAAGCTC TACAAAGGGC CCAACGGGCA GATGACCGTG TTCAGCTTCG 600

TCGCCGAGTC CAACGTGAAC AACTTCAGCG GTGACCTTAA CGCTTTCATC AAGTACCTCA 660

CCGGCAACCA GGGCCTTCCC GCCTCGCAAT ACATCAAGAG CATTGGCGCT GGCACTGAGC 720

CGTTCACGGG TTCCAACGCC AAGCTGACCA CTTCCTCCTA CACTGTCAGC TTCAGATAAC 780

TGTGAAGCTT TATGCTGCCC TTATGCATCA TCCTTGTACA TAGTTATCAC CAGGGGACTC 840

TTGTAAATAC GATTGCCTTA TTAACCGCCT GCATCTGCTT TCACACAATG GCATTTACCA 900

ATCAACAGTG CGCCTCGAAT CCGTAAAAGG TGGCTTAAAA AAAAAAAAAA AAAAAAAAAA 960

AATTCCTGCG GCCGC 975

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 244 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Lys Phe Ser Ser Ala Leu Phe Leu Ala Ala Thr Ala Val Leu Ala
1               5                  10                  15

Ser Ala Ala Pro Leu Glu Arg Arg Ala Asp Phe Cys Gly Gln Trp Asp
            20                  25                  30

Asn Val Lys Asn Gly Pro Tyr Thr Leu Tyr Asn Asn Leu Trp Gly Lys
        35                  40                  45

Asp Ala Ser Gly Ala Ser Gly Ser Gln Cys Thr Gly Val Asp Ser Phe
    50                  55                  60

Ser Ser Asn Thr Ile Ala Trp His Thr Ser Trp Ser Trp Ser Gly Ala
65                  70                  75                  80

Gln Tyr Asn Val Lys Ser Tyr Ala Asn Val Val Val Asp Ile Thr Ser
                85                  90                  95

Lys Lys Leu Ser Ala Ile Ser Ser Ile Asn Ser Ile Trp Arg Trp Ala
            100                 105                 110

Tyr Thr Gly Ser Asn Ile Val Ala Asn Val Ala Tyr Asp Ile Phe Thr
        115                 120                 125

Ser Ser Thr Val Gly Gly Ser Glu Glu Tyr Glu Ile Met Ile Trp Val
    130                 135                 140

Gly Ala Leu Gly Gly Ala Gly Pro Ile Ser Ser Thr Gly Ser Pro Ile
145                 150                 155                 160

Ala Thr Val Ser Leu Ala Gly Ser Ser Trp Lys Leu Tyr Lys Gly Pro
                165                 170                 175

Asn Gly Gln Met Thr Val Phe Ser Phe Val Ala Glu Ser Asn Val Asn
            180                 185                 190

Asn Phe Ser Gly Asp Leu Asn Ala Phe Ile Lys Tyr Leu Thr Gly Asn
        195                 200                 205

Gln Gly Leu Pro Ala Ser Gln Tyr Ile Lys Ser Ile Gly Ala Gly Thr
    210                 215                 220

Glu Pro Phe Thr Gly Ser Asn Ala Lys Leu Thr Thr Ser Ser Tyr Thr
225                 230                 235                 240

Val Ser Phe Arg
```

Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 9, line 17-22

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 448.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 9, line 28 to page 10, line 5

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
2 January 1996	CBS 831.95

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, lines 6-12

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☑

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
12 March 1996	CBS 274.96

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, lines 7-13

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 446.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, lines 20-26

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 444.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 11, line 31 to page 12, line7

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 447.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12, lines 10-16

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 445.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 12, line30 to page 13, line 6.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
2 January 1996	CBS 830.95

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, lines 12-18.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 442.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, lines 23-29.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
23 January 1997	CBS 424.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, line 30 to page 14, line 6.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*
Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
23 January 1997	CBS 425.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 29 to page 16, line 6.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, The Netherlands

Date of deposit	Accession Number
28 January 1997	CBS 443.97

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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Susan Kjellerup

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Applicant's or agent's file reference number 5154-WO	International application No. PCT/DK98/00076

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 17, lines 11-16.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet [X]

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
12 February 1997	DSM 11404

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 45, lines 17-18.

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☐

Name of depositary institution
DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY

Date of deposit	Accession Number
24 February 1998	DSM 12032

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

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CLAIMS

1. A method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising
(a) culturing in a suitable nutrient medium a microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and
(b) subsequently recovering of the XET enzyme from the nutrient medium.

2. The method according to claim 1, wherein the microorganism is a fungus or a bacterium.

3. The method according to claim 2, wherein the fungus is a basidiomycota, an ascomycota, a zygomycota or a mitosporic fungus.

4. The method according to claim 3, wherein the fungus is a basidiomycotinum strain of the order *Coriolales, Schizophyllales, Stereales, or Xenasmatales*.

5. The method according to claim 4, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the families *Coriolaceae, Corticiaceae, Schizophyllaceae, Stereaceae* and *Tubulicrinaceae*.

6. The method according to claim 4, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the genera *Trametes, Corticium, Schizophyllum, Stereum* and *Tubulicrinis*.

7. The method according to claim 6, wherein the fungus is a basidiomycotinum selected from a strain belonging to the group consisting of the species *Trametes hirsuta, Corticium roseum, Schizophyllum sp, Stereum hirsutum* and *Tubulicrinis subulatus*.

8. The method according to claim 7, wherein the fungus is a *Schizophyllum sp*, deposit no. CBS 443.97.

9. The method according to claim 3, wherein the ascomycetes is selected from a strain belonging to the group consisting of the classes *Loculoascomycetes, Discomycetes, Pyrenomycetes,* and *Plectomycetes*.

10. The method according to claim 9, wherein the ascomycetes is selected from a strain belonging to the group consisting of the orders *Dothideales, Rhytismatales*, Pezizales, *Leotiales, Xylariales, Hypocreales, Halosphaeriales, Eurotiales, Phyllachorales* and *Diaporthales*.

11. The method according to claim 10, wherein the ascomycetes is selected from a strain belonging to the group consisting of the families *Leptosphaeriaceae, Botryosphaeriaceae, Dothioraceae, Mycosphaerellaceae, Tubeufiaceae, Rhytismataceae, Sarcosomataceae, Pyronemataceae, Sclerotiniaceae, Amphisphaeriaceae, Hyponectriaceae, Xylariaceae, Valsaceae, Melanconidaceae, Hypocreaceae, Halosphaeriaceae, Phyllachoraceae* and *Trichocomataceae*.

12. The method according to claim 11, wherein the ascomycetes is selected from a strain belonging to the group consisting of the genera *Coniothyrium, Phoma, Diplodia, Plowrightia, Phyllosticta, Septoria, Tubeufia, Alternaria, Embellisia, Tiarosporella, Galiella, Oedocephalum, Pseudoplectania, Pyronema, Botrytis, Aposphaeria, Pestalotia, Pestalotiopsis, Chaetapiospora, Poronia, Nodulisporium, Xylaria, Cytospora, Discula, Phomopsis, Coryneum, Seimatosporium, Fusarium, Verticillium, Volutella, Lulworthia, Colletotrichum, Aspergillus, Eurotium, Eupenicillium, Penicillium, Petromyces* and *Talaromyces*.

13. The method according to claim 12, wherein the ascomycetes is

selected from a strain belonging to the group consisting of the species *Diplodia gossypina*, *Plowrightia ribesia*, *Phyllosticta sp*, *Septoria sp*, *Tubeufia amazonensis*, *Alternaria sp*, *Embellisia hyacinthi*, *Phoma neoloba*, *Phoma tropica*, *Coniothyrium sp*, *Coniothyrium olivaceoum*, *Coniothyrium dunckii*, *Tiarosporella sp*, *Tiarosporella phaseolina*, *Galiella celebica*, *Pseudoplectania nigrella*, *Pyronema domesticum*, *Oedocephalum sp*, *Botrytis cinerea*, *Aposphaeria sp*, *Pestalotia sp*, *Pestalotiopsis sp*, *Poronia punctata*, *Xylaria sp*, *Nodulisporium sp*, *Fusarium solani*, *Verticillium sp*, *Volutella buxi*, *Chaetapiospora rhododendri*, *Lulworthia uniseptata*, *Colletotrichum aculatum*, *Colletotrichum crassipes*, *Cytospora spp*, *Discula sp*, *Phomopsis sp*, *Phomopsis cirsii*, *Coryneum castaneicola*, *Seimatosporium lichenicola*, *Aspergillus tamarii*, *Eurotium chevalieri*, *Eupenicillium javanicum*, *Penicillium capsulatum*, *Penicillium olsonii*, *Penicillium pinophilum*, *Penicillium roqueforti*, *Penicillium italicum*, *Penicillium verruculosum*, *Penicillium canescens*, *Petromyces alliaceus* and *Talaromyces flavus*.

14. The method according to claim 13, wherein the ascomycetes is selected from a strain belonging to the group consisting of the species *Botrytis cinerea* deposit no. CBS 447.97, *Pseudoplectania nigrella* deposit no. CBS 444.97, *Tiarosporella phaseolina* deposit no. CBS 446.97, *Pestalotia sp* deposit no. CBS 445.97 and *Lulworthia uniseptata* deposit no. CBS 442.97.

15. The method according to claim 3, wherein the zygomycota is selected from a strain belonging to the order *Mucorales*.

16. The method according to claim 15, wherein the fungus is a zygomycotum selected from a strain belonging to the group consisting of the families *Chaetocladiaceae* and *Mucoraceae*.

17. The method according to claim 16, wherein the fungus is a zy-

gomycotum selected from a strain belonging to the group consisting of the genera *Dichotomocladium*, *Actinomucor*, *Gongronella*, *Sporodiniella*, and *Mucor*.

18. The method according to claim 17, wherein the fungus is a zygomycota selected from a strain belonging to the group consisting of the species *Dichotomocladium hesseltinei*, *Actinomucor elegans*, *Gongronella butleri*, *Mucor miehei var minor* and *Sporodiniella umbellata*.

19. The method according to claim 18, wherein the fungus is a *Gongronella butleri* deposit no. CBS 448.97.

20. The method according to claim 2, wherein the fungus is a mitosporic fungus.

21. The method according to claim 1, wherein the microorganism is *Vialaea insculpta*.

22. The method according to claim 2, wherein the bacterium is gram-positive.

23. The method according to claim 22, wherein the gram-positive bacterium is *Bacillus*.

24. The method according to claim 23, wherein the gram-positive bacterium is *Bacillus alcalophilus* deposit no DSM 11404.

25. Use of a XET preparation obtained according to any of claims 1-24 for treating a cellulosic material.

26. The use according to claim 25, wherein the treatment provides improved strength and/or improved shape-retention and/or improved anti-wrinkling properties of the cellulosic material.

27. The use according to claim 25, wherein the cellulosic material is a cellulosic fabric or a paper and pulp product.

28. A xyloglucan endotransglycosylase preparation which is producible by cultivation of a microorganism expressing a microbial XET.

29. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 1, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID No. 1.

30. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 2, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID No. 2.

31. The preparation according to claim 28, wherein the XET has the amino acid sequence shown in SEQ ID No. 4, or the XET has an amino acid sequence which is at least 80% homologous with SEQ ID No. 4.

32. The preparation according to claim 28, wherein the XET is active between 3 and 11, in particular between 4 and 9.

33. A method for the production of a xyloglucan endotransglycosylase enzyme (XET) comprising
(a) culturing in a suitable nutrient medium a transformed host microorganism expressing a microbial XET under conditions conducive to the production of the XET enzyme, and
(b) subsequently recovering of the XET enzyme from the nutrient medium.

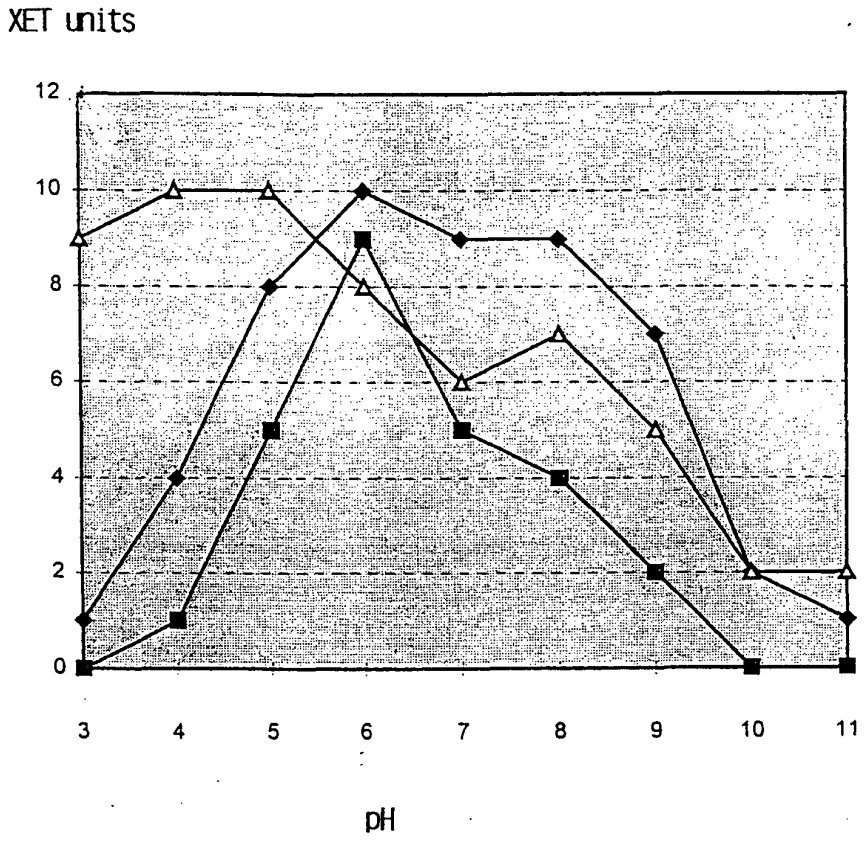

Fig. 1

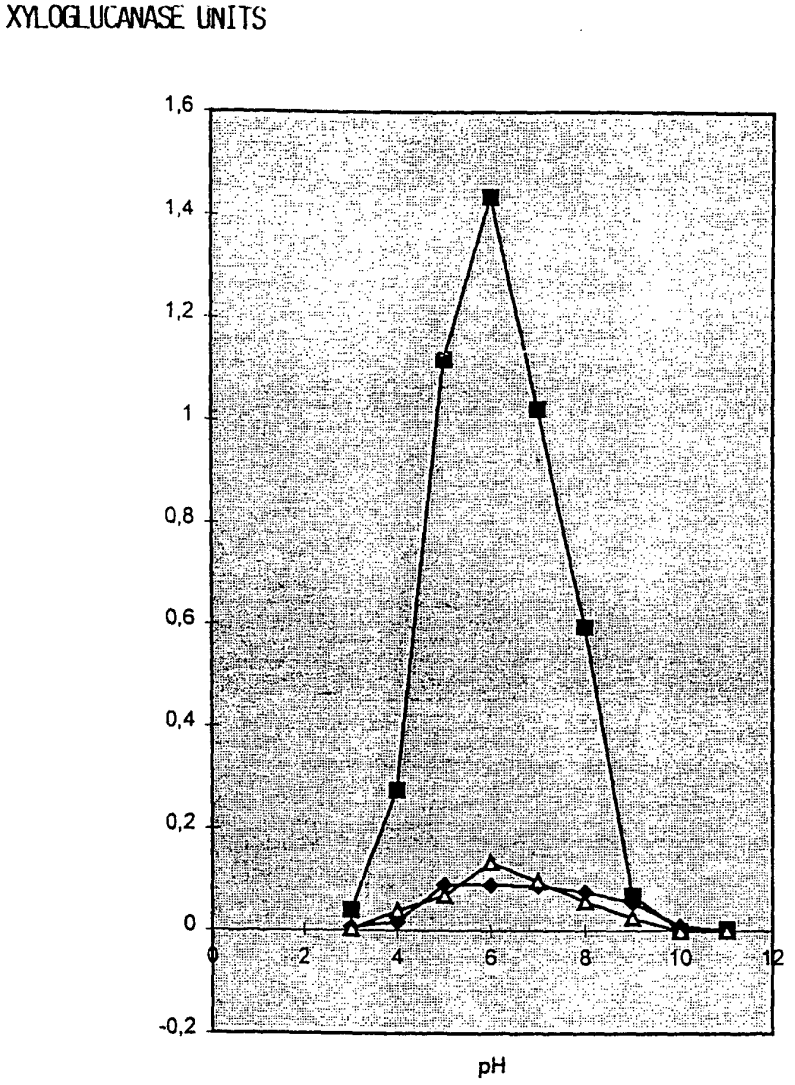

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00076

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12N 9/24, C12N 9/42
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9513384 A1 (UNILEVER PLC), 18 May 1995 (18.05.95)	1-24
X		25-33
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A	EP 0562836 A1 (TAKARA SHUZO CO.LTD.), 29 Sept 1993 (29.09.93)	1-24
X		25-33
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 1998	17 -06- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00076

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Service, File Biosis, Dialog accession no. 7153746, Biosis accession no. 93138746, Fry S. C. et al: "Xyloglucan Endotransglycosylase A New Wall -Loosening Enzyme Activity From Plants", Biochem J 282 (3). 1992. 821-828	1-24
X	-- --------	25-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

29/04/98

International application No.
PCT/DK 98/00076

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		CA 2176133 A	18/05/95
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		HU 74598 A	28/01/97
		HU 9601232 D	00/00/00
		JP 9511121 T	11/11/97
		PL 317046 A	03/03/97
		SK 57696 A	01/10/96
EP 0562836 A1	29/09/93	AU 667706 B	04/04/96
		AU 3540593 A	30/09/93
		CA 2092366 A	27/09/93
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		JP 7079778 A	28/03/95
		US 5516694 A	14/05/96